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Contrasting reproductive strategies of triploid hybrid males in vertebrate mating systems

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Abstract

The scarcity of parthenogenetic vertebrates is often attributed to their 'inferior' mode of clonal reproduction, which restricts them to self-reproduce their own genotype lineage and leaves little evolutionary potential with regard to speciation and evolution of sexual reproduction. Here, we show that for some taxa, such uniformity does not hold. Using hybridogenetic water frogs (*Pelophylax esculentus*) as a model system, we demonstrate that triploid hybrid males from two geographic regions exhibit very different reproductive modes. With an integrative data set combining field studies, crossing experiments, flow cytometry and microsatellite analyses, we found that triploid hybrids from Central Europe are rare, occur in male sex only and form diploid gametes of a single clonal lineage. In contrast, triploid hybrids from north-western Europe are widespread, occur in both sexes and produce recombined haploid gametes. These differences translate into contrasting reproductive roles between regions. In Central Europe, triploid hybrid males sexually parasitize diploid hybrids and just perpetuate their own genotype – which is the usual pattern in parthenogens. In north-western Europe, on the other hand, the triploid males are gamete donors for diploid hybrids, thereby stabilizing the mixed $2n$-$3n$ hybrid populations. By demonstrating these contrasting roles in male reproduction, we draw attention to a new significant evolutionary potential for animals with nonsexual reproduction, namely reproductive plasticity.

Introduction

In vertebrates, a little more than 0.1% of extant species reproduce by parthenogenesis *sensu lato*, that is by apomictic and automictic parthenogenesis, gynogenesis or hybridogenesis (for details see Suomalainen *et al.*, 1987; Parker & Niklasson, 1999; Vrijenhoek, 1999; Neaves & Baumann, 2011). Comparative studies of these reproductive modes are not only important for understanding the evolution of parthenogenesis and explaining the paradox of sex (Otto & Lenormand, 2002), they also yield a deeper understanding of the origin of eukaryotic reproduction and its various pathways (Bengtsson, 2009; rev. in Schön *et al.*, 2009). Parthenogenetic (also called unisexual) vertebrates mostly arose by hybridization between two phylogenetically related sexual species (Vrijenhoek *et al.*, 1989; Avise, 2008; Choleva *et al.*, 2012; but see Sinclair *et al.*, 2010). Combining two different, independently evolving genomes of sexual progenitors leads to difficulties in pairing of divergent homologs during gametogenesis. This has modified the normal meiotic cycle in hybrids so that chromosome segregation and recombination is absent or limited during gametogenesis. Occasionally the meiotic problems also result in the production of diploid gametes which, after fusion with haploid or diploid ones, produce triploid or tetraploid individuals (Stenberg & Saura, 2009). The preconditions and the evolutionary role of polyploidy plays in animal systems are, however, still widely debated (Cunha *et al.*, 2008; Christiansen & Reyer, 2009; Choleva *et al.*, 2012).

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Unisexual reptiles are strictly parthenogenetic, whereas fish and amphibians are sperm-dependent parthenogens. Therefore, the latter can be considered as sexual parasites that must live and mate with an ancestor (the sexual host) to obtain the sperms that are necessary for the parthenogen's reproduction (Vrijenhoek, 1989). In one form, hybridogenesis, the parasitic taxa have a hemiclonal heredity mode, because only one of their parental genomes is transmitted to the next generation, whereas the second parental genome is eliminated prior to meiosis. True syngamy between a haploid clonal gamete (called a hemiclone *sensu* Vrijenhoek, 1979) from the hybridogenetic hybrid and a recombined gamete provided by the parental species whose genome has been eliminated in the hybridogens reconstitutes a hybrid state in the progeny. Therefore, maternal and paternal genomes do not recombine, except on rare occasions (Vorburger, 2001b; Guex *et al.*, 2002; Schmeller *et al.*, 2005; Lamatsch & Stöck, 2009).

The general rareness of unisexual vertebrates is attributed to the necessity to overcome several problems before they can establish themselves within a narrow evolutionary window (so-called balance hypothesis; Moritz *et al.*, 1989). These problems include genetic incompatibilities between nonrelated parental genomes in hybrids, segregation of parental genomes during meiosis and finding an ecological niche in competition with their progenitor species. Another consequence is that most unisexuals maintain a single clonal reproductive mode within a mating complex, irrespective of whether they are of a monophyletic origin (e.g. North American hybrid fish *Poecilia formosa*; Stöck *et al.*, 2010a), or of an ongoing polyphyletic origin (e.g. European hybrid fish of the genus *Cobitis*; Choleva *et al.*, 2008; Janko *et al.*, 2012). Hence, unisexual vertebrates are generally considered as taxa with a low evolutionary potential in terms of speciation and evolution of sex. Their uniform reproductive mode, so the argument, allows for a single role only: to self-reproduce their own genotypes or individual lineages (e.g. Vrijenhoek, 1989; Maynard-Smith, 1992).

This, however, is not always true. Some of these mating systems display reproductive plasticity with signs of an evolutionary potential. This plasticity is achieved through at least two co-occurring factors. First, although hybrid males are usually rare and sterile (e.g. Choleva *et al.*, 2012), functional hybrid males occur regularly in some taxa. These include the hybridogenetic water frog *Pelophylax esculentus* (Graf & Polls Pelaz, 1989; Polls Pelaz, 1994; Christiansen & Reyer, 2009), the fishes *Squalius alburnoides* (Alves *et al.*, 2001) and *Hypseleotris* (Schmidt *et al.*, 2011), and the Palearctic green toad of the *Bufotes viridis* complex (Stöck *et al.*, 2010b). Second, a single hybrid genotype of the above mentioned taxa can often produce more than one type of gametes with some level of recombination between the conspecific genomes in polyploids (e.g. Uzzell *et al.*, 1975; Stöck *et al.*, 2010b, 2012). Together, these two factors may result in dynamic reproductive relationships (Alves *et al.*, 2001). This can lead to the formation of a new bisexual species via polyploid speciation (Cunha *et al.*, 2008), or play a key role in maintaining bisexual hybrid populations by releasing the hybrid from its reproductive dependence on a sexual progenitor (Günther, 1975; Günther *et al.*, 1979; Christiansen & Reyer, 2009).

The *Pelophylax esculentus* study system

In this study, we address the origin and test ambiguous reproductive roles of male polyploidy in *P. esculentus* hybrid water frogs by comparing new results from a detailed investigation on a local scale with previously published results on a wide geographic scale (Pruvost *et al.*, 2013a). The *P. esculentus* complex includes two sexual species, *Pelophylax lessonae* (Camerano, 1882), the pool frog (genotype LL), and *Pelophylax ridibundus* (Pallas, 1771), the marsh frog (RR). From their primary hybridization originated, and still originates, the bisexual hybridogenetic *P. esculentus* (Linnaeus, 1758), the edible frog (genomic composition LR) (Fig. 1a). In most of the species' European range, diploid *P. esculentus* live in sympatry with *P. lessonae*. In these so-called L-E systems, the hybrid excludes its haploid L genome, transmits in its gametes the haploid R genome and restores hybridity in the new generation by obtaining the L genome from mating with *P. lessonae* (Fig. 1b). In some populations, the mirror images, so-called R-E systems, are found. Here, most diploid *P. esculentus* hybrids exclude the R, transmit their L genomes and mate with *P. ridibundus* to perpetuate the hybrid populations (reviewed by Graf & Polls Pelaz, 1989; Plötner, 2005).

In several areas of the species' range also triploid hybrids have been found. This is especially true for northern European regions belonging to the drainage basins of the North Sea and the Baltic Sea (Berger, 1988b; Rybacki & Berger, 2001; Plötner, 2005). In this area, the most frequent population structure is the one with no parental species and two or three types of hybrids: diploid LR in sympatry with triploid hybrids, mostly with LLR, but also with LRR or both (Christiansen & Reyer, 2009; Arioli *et al.*, 2010; Jakob *et al.*, 2010; Pruvost *et al.*, 2013a). In those all-hybrid populations, triploids of both genomic compositions (LLR and LRR) are usually formed by fusion of diploid clonal LR eggs produced by LR females with haploid recombined L or R sperm of LLR or LRR males, respectively (see Fig. 1d for the LR/LLR populations). Diploid hybrids (LR) can arise from the fusion of haploid-recombined L and R gametes of male and female LLR and LRR, respectively, and from the fusion of recombined L eggs of LLR females and haploid clonal R sperm of LR males (for details see Christiansen, 2009 and Christiansen & Reyer, 2009). Therefore, by providing recombined haploid gametes in E-E

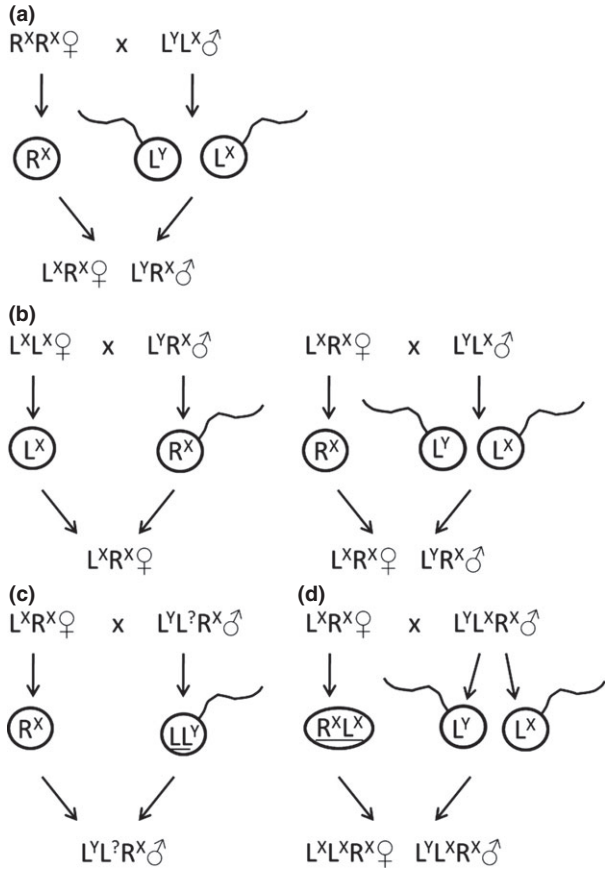

Fig. 1 Origin of (a) diploid *Pelophylax esculentus* (LR) from primary hybridization between *Pelophylax lessonae* males (LL) and *Pelophylax ridibundus* females (RR) and (b) perpetuation of diploid and (c) triploid hybrid lineages in Central Europe and (d) north-western Europe. Gamete types are shown in circles and underlined in case of diploid gametes. X and Y indicate female and male sex-determining factors, respectively.

systems, triploid males substitute the role of parental species in L-E and R-E systems (Günther *et al.*, 1979), turning sperm-dependent hybridogens into independent 'sexually' reproducing units with an evolutionary potential (Christiansen & Reyer, 2009).

In contrast to this pattern, hybrids from Central Europe are mostly of diploid genomic constitution (Berger *et al.*, 1988; Vorburger, 2001a; Plötner, 2005; Mikulíček *et al.*, 2014a). So far, triploids have been reported only from two Central European regions and only in the form of LLR males (Tunner & Heppich-Tunner, 1992; Tunner, 2000; Mikulíček & Kotlík, 2001; Mikulíček *et al.*, 2014a). In contrast to the well-studied species' north-western range, where triploid hybrids flourish, the gamete production pattern and the reproductive role of LLR males in Central Europe were poorly known. It was also not known whether triploids in the two geographical areas originated from the same or different hyridization events.

We, therefore, sampled the area with Central European triploid *P. esculentus* populations to address the origin and heredity mode of LLR males to better understand how polyploid vertebrates can evolve from their sexual ancestors and to investigate whether they use different reproductive modes in different geographic areas. We particularly studied the following four topics: (I) The structure of populations in terms of genotypes, ploidy levels and sex ratios; (II) Gamete types of diploids and triploids, and formation of triploids; (III) The role of triploids within the breeding system; and (IV) Single or multiple origin and nature of hemiclonally transmitted genomes. Here, we integrate multiple types of data from European water frogs to demonstrate contrasting reproductive pathways (self-reproducing mode or contributing to perpetuate the hybrid population) found within a single parthenogenetic mating system (*P. esculentus* complex), genotype and sex.

Materials and methods

To address the origin and role of polyploidy in water frog systems, we combined multiple types of data. We did a comparative field study (for topic I), performed artificial crossing, conducted microsatellite analyses experiments and flow cytometry on sperms (for topics II and III) and compared gamete production patterns, triploid formation and hemiclonal lineages among the eight populations from our study area in Central Europe (for topic IV).

Sampling

During springs 2008–2010, we collected both published and unpublished data on the assumed presence of triploid *P. esculentus* in Central Europe and sampled a total of 524 specimens from eight populations in Slovakia and one in the Czech Republic (see Fig. 2 for locations and Table 1 for names, coordinates, frog sample size and type of each population). We also sampled twice (May 2009 and June 2014) in an area studied by Tunner & Heppich-Tunner (1992) (see ellipse in Fig. 2), but in contrast to these authors, there we did not find a single polyploid frog in a total of more than 200 individuals. Frogs were hand-collected at night and kept separated by sexes in spacious plastic containers. They were assigned to taxa (*P. lessonae*, *P. ridibundus* and *P. esculentus*) according to species-specific morphological characters (Plötner, 2005). All specimens were measured, photographed and toe clipped. Ploidy levels of the *P. esculentus* hybrids were determined by erythrocytes' size in field conditions (erythrocytes of triploids are significantly larger than diploid ones; Berger, 1988a; Vinogradov *et al.*, 1990) and later confirmed by DNA microsatellite analyses in the laboratory. Frogs selected for crossing were individually transpondered (RFID PIT tag AEG ID-162, Ulm, Germany), separated

Table 1 Population types and number of frogs sampled in each of them (*N* LL = number of *Pelophylax lessonae*, *N* LR = number of diploid *Pelophylax esculentus*, *N* LLR = number of triploid LLR *P. esculentus*, *N* RR = number of *Pelophylax ridibundus*).

Country	Population	Abbreviation	Latitude/Longitude	*N* LL	*N* LR	*N* LLR	*N* RR	*N* total	Pop. type
Slovakia	Šprinclov majer	Spri	48°12′59″N/ 17°11′15″E	–	–	–	10 (5/5/0)	10	–
	Borský Mikuláš	Bors	48°37′45″N/ 17°11′17″E	15 (9/6/0)	24 (4/15/5)	–	–	39	1
	Kalaštov	Kala	48°37′55″N/ 17°15′12″E	3 (1/2/0)	32 (2/30/0)	–	–	35	1
	Brodské	Brod	48°41′37″N/ 17°00′29″E	4 (1/2/1)	35 (4/18/13)	–	52 (26/13/13)	91	2
	Šaštín-Stráže	Sast	48°37′55″N/ 17°08′40″E	27 (26/1/0)	79 (31/43/5)	–	26 (15/11/0)	132	2
	Bahno	Bahn	48°37′33″N/ 17°16′24″E	–	31 (??/20/11)	5 (5/0/0)	–	36	3
	Kozí Chrbát	Kozí	48°37′53″N/ 17°17′41″E	–	20 (??/19/1)	52 (40/0/12)	–	72	3
	Šajdíkove Humence	Sajd	48°38′34″N/ 17°16′54″E	–	12 (2/10/0)	20 (15/0/5)	2 (0/0/2)	34	4
Czech Republic	Borovec	Boro	49°38′08″N/ 18°06′01″E	–	50 (30/15/5)	6 (5/0/1)	19 (1/5/13)	75	4
	Total			49 (37/11/1)	283 (73/170/40)	83 (65/0/18)	109 (47/34/28)	524	

Numbers in brackets give the number of males, females and individuals of unknown sex, respectively; ?? indicates that in Bahno and Kozí Chrbát, no LR males were caught, although their presence is likely.

by sex and population of origin and transported to the University of Zürich. During transport, they were stored in cloth bags containing small pieces of rubber sponge and showered daily with fresh water. All frogs survived the journey. Once in Zürich, they were kept separated by sexes, released in outdoor cages and fed *ad libitum* with live crickets.

Artificial crossing experiments

We studied the gamete production pattern of hybrid frogs coming from the populations where triploids were found (Table 1), with the exception of Bahno, because this population was discovered later in the course of this study. Instead, we included one population without triploids (Šaštín-Stráže) where we caught a large number of diploid hybrids of both sexes. The original experimental design was to cross each hybrid both with other hybrids and with at least one specimen of each parental species to determine whether they produce clonal or recombined gametes. Because some females had a limited number of eggs, the full design could not be applied in the populations of Šajdíkove Humence and Borovec (see results in Table 2). Based on results of previous studies using four to eight allozyme markers and crossing experiments with frogs from four Central European populations (Tunner, 1980; Tunner & Heppich-Tunner, 1992; Mikulíček & Kotlík, 2001), we tested with a set of microsatellites a prediction whether diploid and LLR triploid hybrids produce haploid R and diploid LL gametes, respectively. Artificial fertilizations were achieved following the Berger *et al.* (1994) protocol with slight modifications: To induce ovulation, females were injected with 100 μL per 10 g body mass of a 20 mg L^{-1} LHRH hormone in Holtfreter solution (59 mM NaCl, 0.7 mM KCl, 0.9 mM $CaCl_2$, 2.4 mM $NaHCO_3$ and 1.6 mM $MgSO_4$, pH 7.4). Males were anesthetized in a buffered solution of MS-222 (0.15 g L^{-1}) before having one of their testes removed and lacerated into a Petri dish to obtain the sperm solution. This protocol permits the use of the same sperm solution to fertilize eggs from different females and to cross the same female with different males. After about 15 days, the obtained embryos reached free swimming stage (stage 25, Gosner, 1960) and were euthanized using an overdosed MS-222 buffered solution (2 g L^{-1}). The offspring of a few crosses were used for other experiments (Pruvost *et al.*, 2013b), but their genotypic data could also be used for our purpose.

Flow cytometry

Forty-three hybrids were analysed by flow cytometry to confirm their ploidy level and, if males, to determine ploidy level of their sperms. Blood and sperm samples were stabilized in buffer (40 mM citric acid trisodium salt, 0.25 M saccharose and 5% DMSO) and immediately frozen at −80 °C (Cunha *et al.*, 2008). Samples of both parental species were used as a diploid standard. Relative nuclear DNA content was measured using DAPI fluorochrome applying a commercial kit Cystain two Step High Resolution DNA Staining (Partec GmbH,

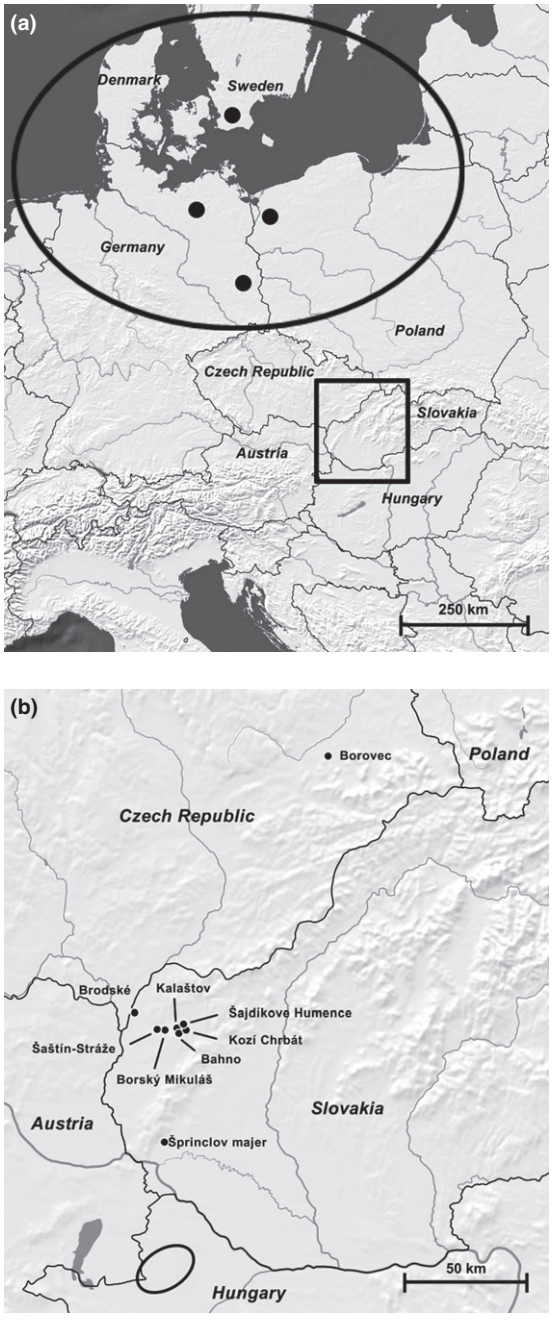

Fig. 2 (a) Areas of water frog populations with triploid hybrids relevant to this study. The ellipse shows the approximate major distribution around the Baltic Sea, with four localities (black dots) for which the north-western European pattern of gamete production has been documented. The rectangle indicates the Central European area investigated in this study. (b) Enlarged map of the study area shows locations of the nine investigated populations (black dots) and a previously studied area (ellipse; Tunner & Heppich-Tunner, 1992) with the same Central European gamete production pattern as found in our study.

Münster, Germany). Fluorescence intensity of 5000 stained nuclei was measured in Partec PA II flow cytometer with a speed 0.5 μL s^{-1}. Flow cytometric histograms were evaluated using FloMax 2.52 software (Partec GmbH, Münster, Germany).

Microsatellite genotyping

The combination of 18 microsatellite loci was used to determine and/or confirm the genomic composition of the crossed specimens and their offspring, in terms of taxon and ploidy level to understand the heredity mode of polyploids. To address the evolution of water frog polyploidy, we used a population genetic approach. Observation of a low genetic diversity and little genetic differentiation within clonally transmitted genomes would suggest a single rather than a multiple origin of hemiclones. DNA was extracted from toe or tail tips of the adult frogs or tadpoles, respectively, stored in 96% ethanol. The Qiagen Biosprint™ 96 DNA Blood Kit (Qiagen, Venlo, the Netherlands) was used for extraction following supplier's protocol. We used a set of 18 microsatellite primer pairs which were run in four primer mixes:

1 Primer Mix 1A – CA1b6, Ga1a19 redesigned (Arioli *et al.*, 2010), RlCA1b5, RlCA5 (Garner *et al.*, 2000), Rrid064A (Christiansen & Reyer, 2009)
2 Primer Mix 1B – Re2CAGA3 (Arioli *et al.*, 2010), Res16, Res20 (Zeisset *et al.*, 2000) RlCA2a34 (Christiansen & Reyer, 2009)
3 Primer Mix 2A – ReGA1a23, Rrid169A, Rrid059A redesigned (Christiansen & Reyer, 2009), Res22 (Zeisset *et al.*, 2000), Rrid013A (Hotz *et al.*, 2001)
4 Primer Mix 2B – Re1CAGA10 (Arioli *et al.*, 2010), RlCA18 (Garner *et al.*, 2000), RlCA1a27, Rrid135A (Christiansen & Reyer, 2009).

Details on PCR protocols are given by Christiansen (2009) and Christiansen & Reyer (2009). Fragment length analysis of the PCR products was run on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ; Life Technologies Europe B.V., Zug, Switzerland), and the alleles were scored with the GeneMapper software 3.7 (Applied Biosystems, Foster City, CA, USA).

We knew from previous studies that three microsatellite loci are species specific for the *P. lessonae* genome (Res20, RlCA1a27 and RlCA18), four are specific for *P. ridibundus* genome (Re2CAGA3, Res22, Rrid169A and Rrid135A), and 11 loci amplify in both the L and R genomes (Christiansen, 2005, 2009; Arioli *et al.*, 2010).

Estimation of null alleles and selection of microsatellite loci

Because L and R genomes do not recombine in hybrids, the two genomes were considered separately in the

Table 2 Origin, genotype, sex and individual numbers of the frogs used in artificial crossing experiments.

Population	Genotype	Sex	Ind. Num.	*N* crosses	*N* offspring	Gametes
Borovec	LLR	M	WFB005-48	3	10	LL
	LR	F	WFB005-41	5	198	R
			WFB005-45	3	100	R
			WFB005-47	5	156	R
		M	WFB005-52	3	106	R
			WFB005-55	3	76	R
Kozí	LLRR	M	WFB015-54	2	32	R
Chrbát	LLR	M	WFB015-55	5	178	LL
			WFB015-56	2	11	LL
			WFB015-57	2	25	LL
			WFB021-16	3	7	LL
			WFB021-17	3	24	LL
			WFB021-18	3	16	LL
	LR	F	WFB021-24	5	104	R
			WFB021-30	3	97	R
Šajdíkove	LLR	M	WFB007-93	4	86	LL
Humence			WFB008-14	3	93	LL
			WFB015-13	4	14	LL
	LR	F	WFB007-91	2	30	R
		M	WFB007-90	2	9	R
Šaštín-	LR	F	WFB007-33	1	8	R
Stráže			WFB007-35	1	12	R
			WFB007-37	4	142	R
			WFB015-72	8	284	R
			WFB015-73	7	161	R
		M	WFB007-52	4	101	R
			WFB007-54	5	79	R
			WFB015-03	6	84	R
			WFB015-04	4	133	R
			WFB015-06	7	254	R

N crosses = number of different partners the individual was crossed with; *N* offspring = number of resulting tadpoles that were analysed; and Gametes = gamete type produced by each individual as deducted from the parents' and the offspring's genotypes. All individuals exclusively produced the indicated gamete type.

subsequent genetic analyses. Prior to these steps, we tested raw data for the presence of null alleles. Nonamplifying loci were rerun for PCR two to three times. When even then no allele was amplified, we attributed the result to the presence of a null allele, rather than to low DNA quality, because this individual DNA amplified for other loci. Potential genotyping errors like stuttering, allelic dropout or presence of null alleles were tested separately for parental RR and LL taxa using the program Micro-Checker 2.2.3 (Van Oosterhout *et al.*, 2004). We estimated frequencies of null alleles with the Brookfield 2 null allele estimator, which treats nonamplifications as data and regards them as null homozygotes when calculating null allele frequencies (Brookfield, 1996). Because this method cannot be applied to the diploid hybrids, we inspected the L and R genomes in hybrids visually and considered the absence of an allele as evidence for a null allele. We then excluded all loci showing an estimated null allele frequency > 0.2 in any of the populations. This led us to exclude locus Re1CAGA10 for the L genome, RlCA2a34 for the R genome, and RlCA5 and Res16 for both genomes from subsequent analyses. We also excluded loci Ga1a19 redesigned, Rrid064A and Rrid059A redesigned for the L genome and locus ReGA1a23 for the R genome, because in all samples they showed only one allele per locus and, thus, provided no variation for the genetic analysis. This left us with 8 loci for the L genome and 11 for the R genome: Res20, RlCA2a34, ReGA1a23, RlCA1a27 and RlCA18 (L genome); Ga1a19 redesigned, Rrid064, Re2CAGA3, Res22, Rrid169A, Rrid059A redesigned, Re1CAGA10 and Rrid135A (R genome); and CA1b6, RlCA1b5 and Rrid013A (for both L and R genomes).

Analysis of genetic diversity and differentiation at individual and population levels

We calculated the gene diversity, corrected for sample size, expressed by the expected heterozygosity (H_e, Nei, 1978), using the program SPAGeDi 1.3 (Hardy & Vekemans, 2002) and the allelic richness (AR) using the program FStat 2.9.3.2 (Goudet, 2001). Genetic differentiation between populations and genotypes was measured using F_{ST} statistics following the method of Weir & Cockerham(1984), which is implemented in the program SPAGeDi 1.3. The program allows the combination of multiple ploidy levels in the same analysis. Concerning genetic diversity, we used two tailed pairwise *t*-tests on the values of H_e for each locus, to test the significance of differences between different frog types, independent of their origin, and we used ANOVAS to look for differences in H_e between population types. Statistical tests were run using the program R 2.15.1 (http://www.r-project.org/). Differences in AR among genomes present in different genotypes were carried out using two-sided permutation tests implemented in FStat.

To test whether R and L genomes present in hybridogenetic hybrids are related to those present in the local parental species, Bayesian assignment programs STRUCTURE 2.3.3 (Pritchard *et al.*, 2000; Falush *et al.*, 2007) and BAPS 5.3 (Corander *et al.*, 2003) were applied. These programs use an iterative approach to assign genotypes into *K* populations without *a priori* knowledge of the population membership of individuals, minimizing Hardy–Weinberg (H-W) and linkage disequilibria within populations. Both parental genomes were analysed separately. Models implemented in both programs assume that loci are unlinked and in H-W equilibrium. These assumptions are unlikely to be met in clonal and hemiclonal hybrid populations because of fixed heterozygosity and linkage of multilocus haplotypes. Therefore, we did not infer the most likely

number of *K*, that is clusters with H-W and linkage equilibria. Instead, only fixed $K = 2$ and $K = 3$ were used, assuming hybrids and a parental species ($K = 2$), and diploid hybrids, triploid hybrids and a parental species ($K = 3$) as the clusters, respectively. Using STUCTURE, admixture and uncorrelated allele models were applied. The analyses were based on runs of 10^6 iterations, following a burn-in period of 100 000 iterations. A series of ten independent runs for each *K* was made with the same parameters to test the accuracy of results. In BAPS, a clustering of groups of individuals was run first, followed by an admixture clustering (Corander & Marttinen, 2006; Corander *et al.*, 2008). The number of iterations that were used to estimate the admixture coefficients for the individuals, and the number of reference individuals from each population, was 200. The number of iterations that were used to estimate the admixture for the reference individuals was set to 20.

Analysis of hemiclonal diversity

As coined by Vrijenhoek (1979), the term 'hemiclone' refers to the clonally transmitted haploid genome, which in our case can be of the L or R type. We determined them by a multilocus genotype (MLG), defined by the identical combination of alleles found in our microsatellite analysis. The same MLG can be, however, found also in two or more unrelated sexual individuals when discrimination power of used molecular markers is low. Therefore, we first calculated two statistics, probability of identity (PI) and probability of identity siblings (PIsibs), that estimate the probability that two individuals randomly chosen from a population have the same MLG on a set of markers (Waits *et al.*, 2001). Both statistics were calculated for both parental species using GenAlEx 6.4 (Peakall & Smouse, 2006). PI and PIsibs for *P. ridibundus* were 5.5×10^{-10} and 2.4×10^{-4}, respectively. PI and PIsibs for *P. lessonae* were 1.6×10^{-7} and 2.3×10^{-3}, respectively. These values are reasonably low (cf. Waits *et al.*, 2001), indicating there is low probability that two *P. ridibundus* or *P. lessonae* individuals share the same MLG on a set of used microsatellites. Following this calculation, we applied a conservative approach and recognized a hemiclone when the same MLG was present in our sample more than three times.

As different hemiclonal gametes may fuse (syngamy) and develop into diploid zygotes on the basis of hybrid × hybrid matings (Hotz *et al.*, 1992), we also searched for possible hemiclonal MLG combinations in the individual genomes of the parental species (LL and RR) and in diploid and triploid hybrids (LR and LLR). Because some triploid LLR hybrids may produce also diploid (LL) hemiclonal gametes (Tunner & Heppich-Tunner, 1992; Mikulíček & Kotlík, 2001), we tested the data for the presence of LL hemiclones as well. To do this, we used GenAlEx 6.4 to concatenate the microsatellite alleles, producing a chain of allele sizes which represent our MLGs. We then compared these MLGs to find whether they were present in other populations under study. The detected MGLs were named after the hemiclone type (L, R, LL), followed by a capital letter attributed in accordance to the descending overall frequency (e.g. L-A = *P. lessonae* hemiclone-A = most frequent L hemiclone). For more details, see Table 6.

Results

Structure of water frog populations (topic I)

The genomic composition of the 524 sampled specimens analysed with 18 microsatellite loci showed that all but one population contained two or three water frog genotypes (DNA microsatellite data are given in Data S1). The exception was the Šprinclov majer locality, where we found only *P. ridibundus*. The exact numbers, including sex ratio for each genotype, are listed in Table 1. Based on their genotype composition, the eight populations were classified into four types (1–4), each represented by two localities. The four populations of types 1 and 2 contained only diploid hybrids, whereas the populations of types 3 and 4 were inhabited also by triploids. In all but two populations, diploid genotypes were always found in both sexes with a male bias in the parental species LL and RR and a female bias in LR hybrids (see totals in Table 1). The two exceptions were Bahno and Kozí Chrbát where no LR males were caught. In contrast, triploid LLR hybrids in the four populations of types 3 and 4 occurred as males only; LLR females were neither caught during this study (Table 1) nor found during previous samplings performed by Mikulíček *et al.* (2014a).

Gamete production (topics II and III)

To identify the heredity mode among hybrids, we performed flow cytometry on sperms of 28 males and genotyped 2216 offspring from 96 crosses through microsatellite analyses. Flow cytometric analysis allowed us to distinguish different ploidy levels among frogs (2*n*, 3*n* and one 4*n* individual) and between parental genotypes (RR and LL) (Data S2a). It also allowed distinguishing between haploid sperms (produced by parental males and 2*n* and 4*n* hybrids) and 2*n* sperms (produced by 3*n* males) (Data S2b). In all these cases, the flow cytometric histograms were clearly nonoverlapping. In contrast, overlapping histograms of blood samples did not allow distinguishing between genotypes of diploid hybrids (LR) and parental species, nor was it possible to tell whether LLR males produced LL or LR sperms. However, in combination with results from the artificial crossing experiment, we unambiguously iden-

tified the gamete production pattern, including for female eggs which cannot be analysed through flow cytometry. All specimens of the two sexual parental species used for the crosses acted as normal haploid gamete donors (L in *P. lessonae*, R in *P. ridibundus*) with chromosome segregation in accordance with the second Mendel's law. Both sexes of LR hybrids produced haploid R gametes only. The triploid LLR hybrid males exclusively produced diploid clonal LL gametes, a pattern supported by two independent analyses: flow cytometry on sperm (Data S2) and microsatellite analyses on parents and offspring from the crossing experiment (Table 2). The only tetraploid LLRR male (WFB015-54 from Kozí Chrbát) produced haploid R sperms and a few diploid cells of unknown genotypic composition.

Population genetics (topic IV)

Genetic diversity and differentiation

The genetic diversity estimates for the L genomes (H_{eL}) and for the R genomes (H_{eR}), are presented in Table 3 and Data S3. Pooled over all eight populations, gene diversity in the *P. lessonae* genome was significantly lower in LLR triploids ($H_{eL} = 0.256$) compared to *P. lessonae* individuals ($H_{eL} = 0.640$, ANOVA, $t_{(7)} = 2.364$, $P = 0.005$) and diploid hybrids ($H_{eL} = 0.608$, $t_{(7)} = 2.364$, $P = 0.011$). No significant differences in H_{eL} were found between the *P. lessonae* genome of diploids and the parental species ($t_{(7)} = 2.364$, $P = 0.111$). For the *P. ridibundus* genome, significant differences in gene diversity were found between *P. ridibundus* ($H_{eR} = 0.631$) and both LR ($H_{eR} = 0.414$, $t_{(10)} = 2.228$, $P = 0.001$) and LLR hybrids ($H_{eR} = 0.413$, $t_{(10)} = 0.228$, $P = 0.006$), but not between diploid and triploid hybrids ($t_{(10)} = 0.228$, $P = 0.996$). Significant differences in gene diversity between different population types were not observed.

In terms of AR, highly significant differences in the *P. lessonae* genome have been found between LLR (AR = 1.625) and LL (AR = 8.125, two-sided permutation test, $P = 0.0001$), and between LLR and LR (AR = 7.272, $P = 0.003$), but not between diploid hybrids and *P. lessonae* ($P = 0.308$). For the *P. ridibundus* genome, highly significant differences have been found between RR (AR = 8.760) and LR (AR = 4.128, $P = 0.009$), and between RR and LLR (AR = 3.000, $P = 0.002$), but not between diploid and triploid hybrids ($P = 0.825$).

Global F_{ST} values showed significant and substantial differentiation among populations for both genomes. The mean F_{ST} values were 0.271 for the L genome and 0.114 for the R genome, respectively. For the L genomes, we found little genetic differentiation between LL and LR individuals ($F_{ST} = 0.021$), but very large differentiation between LLR and both LL and LR individuals ($F_{ST} = 0.388$ and 0.362 respectively; Table 4). For R genomes, the genetic differentiation was small between LR and LLR hybrids ($F_{ST} = 0.019$), whereas it was large between RR and both LR and LLR hybrids ($F_{ST} = 0.133$ and $F_{ST} = 0.129$, respectively). Pairwise F_{ST} values clearly separated the L genomes of triploid LLR hybrids from those of LR and LL individuals (Table 5). Hence, the triploids were in their L genomes genetically not only strongly differentiated from the parental LL individuals, but also from the diploid LR hybrids in syntopic populations. In contrast, there was little to only moderate genetic differentiation in L genomes of parental LL individuals and diploid LR hybrids in all population types. The only exception was represented by diploid LR hybrids from the Czech population of Borovec, whose L genome was distinct from all other populations (Table 5).

Concerning the R genomes, parental RR individuals from different localities revealed mostly little to large genetic differentiation between themselves and mostly moderate to large differentiation between them and both diploid and triploid hybrids from all population types (Table 5). In contrast, there was only little to moderate differentiation among R genomes of both

Table 3 Gene diversity (H_e) corrected for sample size (Nei, 1978) for *Pelophylax lessonae* genomes (H_L) and *Pelophylax ridibundus* genomes (H_R) in the different frog genotypes (LL, LLR, LR, RR). Sample size is given in brackets.

		H_L			H_R		
Population type	Population name	LL	LLR	LR	LLR	LR	RR
	All populations	0.640 (49)	0.256 (83)	0.608 (283)	0.413 (83)	0.414 (283)	0.631 (109)
PT1 (LL + LR)	Borský Mikuláš	0.650 (15)	–	0.586 (24)	–	0.385 (24)	–
PT1 (LL + LR)	Kalaštov	0.600 (3)	–	0.574 (32)	–	0.418 (32)	–
PT2 (LL + LR + RR)	Brodské	0.594 (4)	–	0.577 (35)	–	0.436 (35)	0.656 (52)
PT2 (LL + LR + RR)	Šaštín-Stráže	0.618 (27)	–	0.558 (79)	–	0.396 (79)	0.602 (26)
PT3 (LLR + LR)	Bahno	–	0.278 (5)	0.590 (31)	0.436 (5)	0.425 (31)	–
PT3 (LLR + LR)	Kozí Chrbát	–	0.252 (52)	0.495 (20)	0.424 (52)	0.429 (20)	–
PT4 (LLR + LR + RR)	Šajdíkove Humence	–	0.256 (20)	0.536 (12)	0.414 (20)	0.275 (12)	0.439 (2)
PT4 (LLR + LR + RR)	Borovec	–	0.273 (6)	0.225 (50)	0.115 (6)	0.029 (50)	0.496 (19)
RR	Šprinclov majer	–	–	–	–	–	0.549 (10)

hybrid types (LR and LLR) from all populations. Again, the population in Borovec stood out, because both the diploid and the triploid hybrids genetically differed in their R genome from parental RR individuals and hybrids found elsewhere.

The results of the two Bayesian programs were concordant and revealed substantial structuring in the *P. lessonae* genome (Fig. 3a). Triploid hybrids on the one hand and diploid hybrids and *P. lessonae* on the other were unequivocally assigned to two separate clusters assuming $K = 2$. Assuming $K = 3$, Bayesian clustering was very similar, with the exception of LR hybrids from Borovec – most of them were assigned to a separate cluster with high probability. Structuring in the *P. ridibundus* genome between the genotypes RR, LR and LLR was not so straightforward (Fig. 3b). More than 90% of *P. ridibundus* individuals were assigned to the cluster 1 regardless of the number of expected K, whereas 64% of both diploid and triploid hybrids were assigned to cluster 2 (including almost all diploid LR from Borovec); remaining hybrids were assigned to the cluster 1 (assuming $K = 2$) and clusters 1 or 3 (assuming $K = 3$). Only few individuals were assigned to more than one cluster revealing admixture across analyses.

Table 4 Pairwise F_{ST} values for L (below the diagonal) and R (above the diagonal) genomes between the hybrid types listed in the left column (LLR, LR) and the hybrids and parental species shown in the top horizontal row (LL, LLR, LR, RR) and parental species (LL, RR), pooled over all populations.

F_{ST}	LL	LLR	LR	RR
LLR	0.388	x	0.019	0.129
LR	0.021	0.362	x	0.133

Analysis of hemiclonal diversity

With respect to the R genomes present in hybrid individuals, we detected a total of 14 hemiclones with different relative frequencies among populations (Table 6 and Data S4). In the Czech population of Borovec, we found only a single hemiclone (R-B), whereas the Slovakian populations contained multiple R hemiclones, ranging from four in Brodské to eight in Šaštín-Stráže (Table 6). Hemiclone R-A occurred in all four population types (PT1-4); five (R-F, R-H, R-K, R-L and R-N) occurred only in populations with parental LL frogs (PT1 and/or PT2); and three hemiclones (R-B, R-

Table 5 Pairwise F_{ST} value comparisons between all genotype-population combinations for L (below the diagonal) and R (above the diagonal) genomes. Darker colours correspond to lower F_{ST} values. For abbreviations of population names, see Table 1.

	Bors LL	Kala LL	Brod LL	Sast LL	Bahn LLR	Kozi LLR	Sajd LLR	Boro LLR	Bors LR	Kala LR	Brod LR	Sast LR	Bahn LR	Kozi LR	Sajd LR	Boro LR	Brod RR	Sast RR	Sajd RR	Boro RR	Spri RR
Bors LL	X																				
Kala LL	.108	X																			
Brod LL	.070	.106	X																		
Sast LL	.009	.052	.043	X																	
Bahn LLR	.280	.492	.470	.788	X	.000	.000	.273	.003	.035	.003	.000	.000	.000	.000	.745	.089	.104	.060	.130	.144
Kozi LLR	.423	.592	.584	.403	.000	X	.000	.208	.085	.045	.109	.085	.004	.000	.098	.424	.143	.177	.132	.180	.200
Sajd LLR	.366	.571	.557	.348	.000	.000	X	.220	.035	.093	.040	.016	.000	.000	.025	.544	.137	.152	.106	.171	.195
Boro LLR	.291	.507	.490	.289	.111	.111	.111	X	.312	.302	.252	.258	.193	.245	.423	.181	.197	.247	.537	.185	.360
Bors LR	.022	.166	.067	.000	.340	.478	.427	.349	X	.150	.049	.048	.054	.063	.052	.585	.166	.173	.154	.215	.237
Kala LR	.102	.127	.135	.100	.302	.432	.381	.313	.127	X	.194	.185	.045	.028	.220	.544	.154	.176	.251	.203	.182
Brod LR	.045	.130	.023	.024	.325	.455	.403	.334	.035	.086	X	.038	.062	.093	.063	.492	.144	.142	.120	.186	.207
Sast LR	.066	.112	.097	.015	.318	.417	.371	.325	.031	.104	.030	X	.061	.084	.059	.431	.162	.156	.149	.206	.222
Bahn LR	.077	.000	.076	.041	.315	.450	.397	.326	.079	.089	.069	.071	X	.000	.054	.448	.140	.154	.146	.155	.201
Kozi LR	.142	.159	.122	.112	.450	.560	.523	.460	.116	.213	.125	.140	.127	X	.070	.566	.137	.165	.130	.174	.197
Sajd LR	.113	.036	.072	.087	.396	.515	.482	.410	.140	.029	.077	.105	.041	.147	X	.747	.215	.232	.231	.255	.329
Boro LR	.392	.528	.535	.354	.709	.696	.702	.708	.431	.419	.389	.370	.397	.537	.487	X	.346	.445	.881	.413	.667
Brod RR																	X	.025	.079	.077	.044
Sast RR																		X	.118	.103	.058
Sajd RR																			X	.180	.191
Boro RR																				X	.168
Spri RR																					X

differentiation	L	FST	R
little		<0.050	
moderate		0.050–0.150	
great		0.150–0.250	
very great		>0.250	

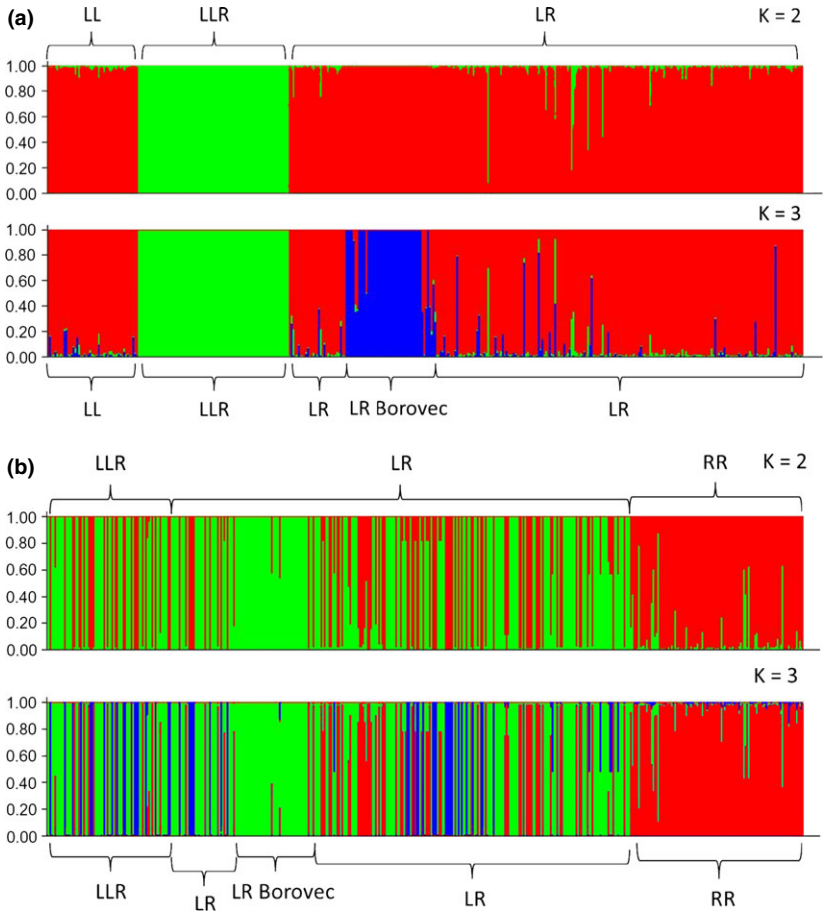

Fig. 3 Structuring in the L genome (a) and the R genome (b) according to a Bayesian analysis assuming two (K = 2) and three (K = 3) clusters, respectively.

G, and R-M) were found only in populations where triploid LLR hybrids are present (PT3 and PT4). The remaining four hemiclones (R-C, R-E, R-I and R-J) were not specific to any population type.

Concerning the L genome, the number of hemiclones was much smaller than within the R genome. We detected only a single L hemiclone (L-A) and two LL (diploid) hemiclones (LL-A and LL-B). L-A occurred only in diploid hybrids from Borovec but there in a very high proportion (38 of 50 sampled LR frogs). Both LL hemiclones were present in all male LLR triploid hybrids (N = 83). One hemiclone (LL-B) was restricted to Borovec, and the other one (LL-A) was present in the three Slovak populations (Bahno, Kozí Chrbát and Šajdíkove Humence). The two LL hemiclones differed by only one allele in their MLG comparison, showing one dinucleotide repetition difference at the locus RlCA18. In Borovec, the locus RlCA18 amplified for alleles 177 and 181, whereas LLR frogs from Slovakia carried alleles 179 and 181. We further found that 35 of 50 LR hybrids from Borovec likely originated from a combination of two hemiclones, namely L-A and R-B (called as Comb-A, Table 6). Triploid LLR individuals in Kozí Chrbát, Šajdíkove Humence and Bahno had also genomes combined from two hemiclones, namely as a combination of LL-A hemiclone and one of six R hemiclones (Comb-B to G, Table 6). Three LLR males from Borovec were composed of LL-B and R-B hemiclones (Comb-H, Table 6).

Discussion

Population composition and gamete production (topics I and II)

In the nine sampling sites that we studied in Central Europe, we have identified four population types, three where hybrids live in sympatry with one or both parental species and one with hybrids only. Our combined data from flow cytometry, crossing experiments, analysis of genetic diversity and gene flow between genotypes show that even in the two apparent all-hybrid populations of type 3, the parental species *P. lessonae* rather than triploid hybrids provide L (*lessonae*) genomes for a new generation of LR hybrids. This is further supported by the F_{ST} statistics and clustering of L genomes from LR hybrids with *P. lessonae* and not with LLR hybrids (Tables 4 and 5),

Table 6 Multilocus genotypes (MLGs) for L, R and LL hemiclones and their combinations found in the study and their distribution over the populations.

Hemiclonal MLGs	Hemiclonal MLG name	Distribution of hemiclonal MLGs in populations	*N* Tot.
R hemiclone in LR and LLR hybrids	R-A	17 Sast (17 LR), 13 Kozi (9 LLR, 4 LR), 10 Sajd (4 LLR, 6 LR), 6 Bahn (2 LLR, 4 LR), 5 Brod (LR), 3 Bors (LR), 2 Kala (LR)	56
	R-B	50 Boro (3 LLR, 47 LR), 2 Bahn (LR), 1 Kozi (LLR)	53
	R-C	21 Kozi (15 LLR, 6 LR), 14 Kala (LR), 8 Bahn (1 LLR, 7 LR), 4 Sajd (3LLR, 1 LR)	47
	R-D	10 Bors (LR), 8 Sast (LR), 7 Kozi (4 LLR, 3 LR), 6 Bahn (LR), 6 Brod (LR), 5 Sajd (2 LLR, 3 LR), 1 Kala (LR)	43
	R-E	14 Kozi (10 LLR, 4 LR), 7 Bahn (2 LLR, 5 LR), 6 Sajd (5 LLR, 1 LR), 2 Kala (LR)	29
	R-F	18 Sats (LR), 1 Kala (LR)	19
	R-G	11 Kozi (8 LLR, 3 LR), 1 Sajd (LLR)	12
	R-H	6 Bors (LR), 5 Sast (LR), 1 Kala (LR)	12
	R-I	8 Kala (LR), 1 Bahn (LR), 1 Bors (LR)	10
	R-J	5 Sast (LR), 3 Brod (LR), 1 Bahn (LR)	9
	R-K	8 Sast (LR)	8
	R-L	6 Sast (LR), 1 Bors (LR)	7
	R-M	5 Kozi (LLR), 1 Sajd (LLR)	6
	R-N	2 Sast (LR), 1 Bors (LR) 1 Brod (LR)	4
	Single MLGs		51
L hemiclone in LR hybrids,	L-A	38 Boro	38
	Single MLGs		245
LL hemiclone in LLR hybrids	LL-A	52 Kozi (LLR), 20 Sajd (LLR), 5 Bahn (LLR)	77
	LL-B	6 Boro (LLR)	6
	Single MLGs		0
Comb. LL+R hemiclones in LLR hybrids	Comb-B	15 Kozi, 3 Sajd, 1 Bahn (composed of LL-A + R-C)	19
	Comb-C	10 Kozi, 5 Sajd, 2 Bahn (composed of LL-A + R-E)	17
	Comb-D	9 Kozi, 4 Sajd, 2 Bahn (composed of LL-A + R-A)	15
	Comb-E	8 Kozi. 1 Sajd (composed of LL-A + R-G)	9
	Comb-F	4 Kozi, 2 Sajd (composed of LL-A + R-D)	6
	Comb-G	5 Kozi, 1 Sajd (composed of LL-A + R-M)	6
	Comb-H	3 Boro (composed of LL-B + R-B)	6
	Single MLGs		11
Comb L+R hemiclones in LR hybrids	Comb-A	35 Boro (composed of L-A + R-B)	35
	Single MLGs		248

Letters (A-N) behind the genomes indicate different hemiclones: 'single MLG' refers to allele combinations that were found in only one or two copies and, hence, were not considered to form a hemiclone. For abbreviations of population names, see Table 1.

as well as by contrasting levels of genetic diversity (H_e and AR), which is comparable between LR hybrids and *P. lessonae* but substantially lower in the LL genome of LLR triploids. L genome provisioning through *P. lessonae* is characteristic for L-E system (here represented by the population type 1), where diploid hybrids clonally transmit R genomes (see Table 2) and receive L gametes from *P. lessonae* (Tunner, 1974; Uzzell & Berger, 1975; Graf & Polls Pelaz, 1989). Even where parental *P. ridibundus* individuals exist, as in population types 2 and 4, they do not seem to be the major contributors of R gametes to hybrid progeny. This is indicated by the fact that genetic differentiation among R genomes (F_{ST}, Bayesian analysis) is larger between LR hybrids and RR sexuals in the same population than between RR individuals sampled in different sites (Table 5; cf. Mikulíček *et al.*, 2014b). Moreover, the R genome of hybrids reveals lower genetic diversity in comparison to *P. ridibundus*, thus showing that only part of the *P. ridibundus* individuals contributed to the formation of hybridogenetic lineages. Our combined data show that LLR hybrids exclusively produce diploid LL rather than haploid L sperms. Thus, matings between LLR males and LR females will result in LLR offspring. This raises the question how then diploid LR hybrids are produced and maintained in the four populations of types 3 and 4. At present, the answer remains open, but we develop three not mutually exclusive hypotheses in Data S5.

The population data and gamete production modes are in agreement with an XX–XY sex determination system in which the hemiclonal genome may be coupled with either an X or Y haploid set of chromosomes (Graf & Polls Pelaz, 1989). In hybrids, the R genome likely carries a female determining factor (X), whereas L genomes carry female (X) or male (Y) determining factors with equal probability (Berger *et al.*, 1988; Christiansen, 2009). Therefore, in principle, when the diploid LL sperms of LLR males fertilize haploid R eggs of LR females, only LLR males ($L^YL^?R^X$) will be produced (Fig. 1c). In contrast, diploid LR hybrids come in both sexes, but with an excess of females because LR males sire daughters only (cf. Fig. 1b). This female bias seems to be particularly extreme in the Pannonian Basin to which all but one (Borovec) study population belongs. In this basin, male proportions as low as 3% have been found (Tunner & Dobrowsky, 1976; Berger *et al.*, 1988; Gubanyi & Creemers, 1994; Mikulíček & Kotlík, 2001). Therefore, the virtual absence of LR males in Bahno and Kozí Chrbát may be the result of a sampling bias that is due to low abundance. In contrast, the lack of LLR females in Central Europe is to be expected from the gamete production pattern (Fig. 1c).

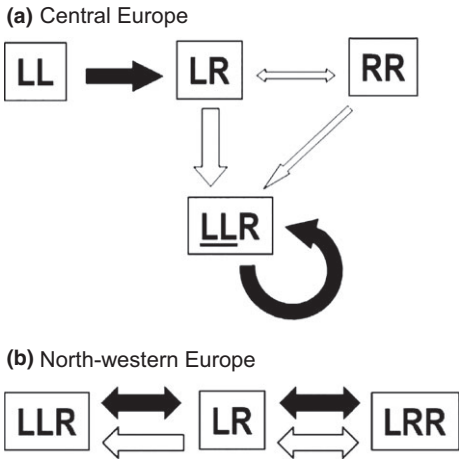

Fig. 4 Heredity pathways of L genomes (black arrows) and R genomes (white arrows) between different genotypes in populations with triploid hybrids from (a) Central Europe (this study) and (b) north-western Europe (simplified from Som & Reyer, 2006; Christiansen, 2009).

The reproductive role of triploid males in Central and north-western Europe (topic III)

In none of the nine Central European populations that we studied in this paper did we find evidence for the type of all-hybrid populations that are typical for north-western Europe. Even the two populations containing only diploid LR and triploid LLR hybrids (type 3 in Table 1) are basically L-E systems. Although both regions share a presence of LLR males, they differ in several aspects which are summarized in Table 7.

First, sex ratios differ markedly among LLR triploids. Because of the XX/XY sex determining mechanism described above, fusion of LL sperms from LLR males and R eggs from LR females in Central Europe will result in LLR males only ($L^YL^?R^X$) (Fig. 1c). In contrast, fusion of haploid L sperms from LLR males with diploid LR eggs from LR females in north-western Europe will produce LLR offspring of both sexes ($L^YL^?R^X$, $L^XL^XR^X$) (Fig. 1d).

Second, LLR males in Central Europe sexually parasitize LR females for self-reproduction, because the resulting progeny are 100% LLR males which will exclude the R genomes at gametogenesis (Fig. 4a). These diploid LR females, in turn, are also sexual parasites because for successful reproduction of hybrid offspring they require a donor of L gametes, which likely comes from *P. lessonae* from neighbouring ponds (Fig. 4a). In contrast, in all-hybrid E-E populations from the north-western Europe, L and R alleles are passed on between diploid and triploid males and females (Berger, 1988b; Günther & Plötner, 1990; Som & Reyer, 2006) (Fig. 4b). In these latter populations, LLR males are sexual hosts for the diploid LR females and, hence, fulfil the key role that *P. lessonae* has in L-E systems. Thus, in north-western Europe, triploids help in stabilizing (all-hybrid) populations by substituting the role of sexual species, whereas in Central Europe, they do not.

Table 7 Differences between water frog populations with triploid LLR individuals in north-western and Central Europe; occasional deviations from this pattern do occur, based on this study and data from Christiansen (2009) Christiansen & Reyer (2009) and Jakob *et al.* (2010).

Features of LLR frogs	Central Europe	North Western Europe
Abundance	Rare	Frequent
Gamete production	Clonal LL gametes	Recombined L gametes
Sex composition	Only males	Both sexes
Origin of triploids	LL sperms from LLR males × R eggs from LR females	L sperms from LLR males × LR eggs from LR females
Reproductive role	Self-reproduction sexual parasites	L gamete donor substitute sexual hosts

The origin of male polyploidy in Central Europe (topic IV)

Our results from microsatellite genotyping, crossing experiments and population genetic statistics consistently indicate that LLR from all populations were very similar with respect to the multilocus genotype (MLG) of their two *lessonae* genomes: in the three Slovakian populations, the MLG was identical, and in the Czech population of Borovec (130 km apart), it differed by only a single allele mutation at the locus RlCA18. We therefore believe that LL hemiclones represent a single clonal lineage which diversified by mutation after hemiclone formation.

The geographic origin of this LL hemiclonal lineage, however, remains puzzling. Given the high genetic differentiation in L genomes between the LLR triploids and the group of Slovakian sexual LL and hybrid LR frogs, the origin is unlikely to have been *in situ*, at least not in a recent time. On the other hand, the LL hemiclone of triploid males is genetically more similar to the L genome of diploid hybrids in Borovec than to the L genome of diploid hybrids in Slovakia. Therefore, we suggest that the LL hemiclonal lineage might have originated somewhere in the area of Borovec, a sample site situated in the proximity of the European watershed of the Baltic Sea, the North Sea and the Black Sea. Subsequently, it may have spread southerly through the Danubian Basin. The origin of the haploid L hemiclone found in LR hybrids in Borovec remains unclear, because we were not able to recognize its donor in the population.

The presence of several R hemiclones in the Slovak populations suggests their multiple origins. This pattern has also been documented for other populations of water frogs (e.g. Tunner, 1974; Uzzell & Berger, 1975; Hotz *et al.*, 2008). Instead of a scenario of ongoing primary hybridizations between *P. lessonae* and *P. ridibundus*, we suppose the existence of several R hemiclones to be explained rather by past than current primary hybridization events. If primary hybridization was ongoing and common, then we would expect low genetic differentiation in R genome between sexual (RR) and hybrid (LR) genomes, i.e. primary hybridization should tend to decrease genetic differentiation between sexual and hemiclonal genomes. Contrary to this expectation, we have found substantial genetic differentiation (i.e. low gene flow rate) between both genomes, corroborating results based on AFLP markers (Mikulíček *et al.*, 2014b).

General evolutionary implications

Hybrid water frog triploids in north-western Europe and in the Central European area represent independent and currently nonrelated evolutionary units characterized by contrasting inheritance modes. At present, we do not know whether the two geographic regions represent single or multiple hybrid origin. However, our results strongly suggest that parthenogenetic animals (*sensu lato*) originating from the same parental species and carrying even the same genotype (here LLR) can independently develop various reproductive roles. These findings place hybrid water frogs in contrast to most other vertebrate parthenogenetic systems. For example, all taxa of parthenogenetic reptiles are virtually constrained into a single reproductive mode, because DNA content in their eggs represents a genetic copy of the mother (see a list of taxa in Kearney *et al.*, 2009). Similarly, most parthenogenetic fish (either diploid or polyploid) show a uniform reproductive system, for example in the genus *Cobitis*, *Poecilia*, *Poeciliopsis* and others (Lamatsch & Stöck, 2009). Although some fish from the *S. alburnoides* complex produce eggs of various ploidies within a single genotype and individual, their role in a mating system is rather complex than contrasting (Alves *et al.*, 2001). Fertile diploid and triploid hybrid males in *Squalius* maintain only clonal spermatogenesis, whereas tetraploids produce one type of meiotic sperms (Collares-Pereira *et al.*, 2013). Therefore, a demonstration of contrasting roles in reproduction of a single genotype in vertebrate parthenogens in general, and in male sex in particular (i.e. to be a donor of gametes vs. to be a sexual parasite as we evidenced in LLR triploids), gives an example of a new significant evolutionary potential (reproductive plasticity) in animals with nonsexual reproduction. The present data also open research questions for future studies, namely how these triploid male lineages with different inheritance modes evolutionarily affect the dynamics of hybrid populations and what happens in a contact zone between the two geographic regions' populations where the two lineages may meet in the same population.

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References

Alves, M.J., Coelho, M.M. & Collares-Pereira, M.J. 2001. Evolution in action through hybridisation and polyploidy in an Iberian freshwater fish: a genetic review. *Genetica* **111**: 375–385.

Arioli, M., Jakob, C. & Reyer, H.-U. 2010. Genetic diversity in water frog hybrids (*Pelophylax esculentus*) varies with population structure and geographic location. *Mol. Ecol.* **19**: 1814–1828.

Avise, J.C. 2008. *Clonality – The Genetics, Ecology, and Evolution of Sexual Abstinence in Vertebrate Animals*. Oxford University Press, New York, NY.

Bengtsson, B.O. 2009. Asex and evolution: a very large-scale overview. In: *Lost Sex. The Evolutionary Biology of Parthenogenesis* (I. Schön, K. Martens & van Dijk P., eds), pp. 1–19. Springer, Dordrecht, the Netherlands.

Berger, L. 1988a. Principles of studies of European water frogs. *Acta Zool. Cracov.* **31**: 563–580.

Berger, L. 1988b. An all-hybrid water frog population persisting in agrocenoses of central Poland (Amphibia, Salientia, Ranidae). *Proc. Acad. Nat. Sci. Philadelphia* **140**: 202–219.

Berger, L., Uzzell, T. & Hotz, H. 1988. Sex determination and sex-ratios in Western Palearctic water frogs – XX and XY female hybrids in the Pannonian Basin. *Proc. Acad. Nat. Sci. Philadelphia* **140**: 220–239.

Berger, L., Rybacki, M. & Hotz, H. 1994. Artificial fertilization of water frogs. *Amphib.-Reptil.* **15**: 408–413.

Brookfield, J.F.Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. *Mol. Ecol.* **5**: 453–455.

Choleva, L., Apostolou, A., Rab, P. & Janko, K. 2008. Making it on their own: sperm-dependent hybrid fishes (*Cobitis*) switch the sexual hosts and expand beyond the ranges of their original sperm donors. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**: 2911–2919.

Choleva, L., Janko, K., De Gelas, K., Bohlen, J., Slechtova, V., Rabova, M. *et al.* 2012. Synthesis of clonality and polyploidy in vertebrate animals by hybridization between two sexual species. *Evolution* **66**: 2191–2203.

Christiansen, D.G. 2005. A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. *Mol. Ecol. Notes* **5**: 190–193.

Christiansen, D.G. 2009. Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). *BMC Evol. Biol.* **9**: 135.

Christiansen, D.G. & Reyer, H.-U. 2009. From clonal to sexual hybrids: genetic recombination via triploids in all-hybrid populations of water frogs. *Evolution* **63**: 1754–1768.

Collares-Pereira, M.J., Matos, I., Morgado-Santos, M. & Coelho, M.M. 2013. Natural pathways towards polyploidy in animals: the *Squalius alburnoides* fish complex as a model system to study genome size and genome reorganization in polyploids. *Cytogent. Genome Res.* **140**: 97–116.

Corander, J. & Marttinen, P. 2006. Bayesian identification of admixture events using multi-locus molecular markers. *Mol. Ecol.* **15**: 2833–2843.

Corander, J., Waldmann, P. & Sillanpää, M.J. 2003. Bayesian analysis of genetic differentiation between populations. *Genetics* **163**: 367–374.

Corander, J., Marttinen, P., Sirén, J. & Tang, J. 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics* **9**: 539.

Cunha, C., Doadrio, I. & Coelho, M.M. 2008. Speciation towards tetraploidization after intermediate processes of non-sexual reproduction. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**: 2921–2929.

Falush, D., Stephens, M. & Pritchard, J.K. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Mol. Ecol. Notes* **7**: 574–578.

Garner, T.W.J., Gautschi, B., Röthlisberger, S. & Reyer, H.-U. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. *Mol. Ecol.* **9**: 2173–2175.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with note on identification. *Herpetologica* **16**: 183–190.

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (2.9.3.2.). http://www2.unil.ch/popgen/softwares/fstat.htm.

Graf, J.D. & Polls Pelaz, M. 1989. Evolutionary genetics of the *Rana esculenta* complex. In: *Evolution and Ecology of Unisexual Vertebrates*, Vol. 466 (R.M. Dawley & J.P. Bogart, eds), pp. 289–301. New York State University Museum Bulletin, Albany, NY.

Gubanyi, A. & Creemers, R. 1994. Population structure of water frogs in a flood plain of the river Danube (Szigetköz) in Hungary. *Zool. Pol.* **39**: 441–445.

Guex, G.-D., Hotz, H. & Demlitsch, R.D. 2002. Deleterious alleles and differential viability in progeny of natural hemiclonal frogs. *Evolution* **56**: 1036–1044.

Günther, R. 1975. Zum natürlichen Vorkommen und zur Morphologie triploider Teichfrösche "*Rana esculenta*", L., in der DDR (Anura, Ranidae). *Mitt. Zool. Mus. Berlin* **51**: 145–158.

Günther, R. & Plötner, J. 1990. Mating pattern in pure hybrid populations of water frogs, *Rana* kl. *esculenta* (Anura, Ranidae). *Alytes* **8**: 90–98.

Günther, R., Uzzell, T. & Berger, L. 1979. Inheritance patterns in triploid *Rana "esculenta"* (Amphibia, Salientia). *Mitt. Zool. Mus. Berlin* **55**: 35–57.

Hardy, O.J. & Vekemans, X. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Mol. Ecol. Notes* **2**: 618–620.

Hotz, H., Beerli, P. & Spolsky, C. 1992. Mitochondrial-DNA reveals formation of nonhybrid frogs by natural matings between hemiclonal hybrids. *Mol. Biol. Evol.* **9**: 610–620.

Hotz, H., Uzzell, T., Guex, G.-D., Alpers, D., Semlitsch, R.D. & Beerli, P. 2001. Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. *Zoosyst. Evol.* **77**: 43–50.

Hotz, H., Guex, G.-D., Beerli, P., Semlitsch, R.D. & Pruvost, N.B.M. 2008. Hemiclone diversity in the hybridogenetic frog *Rana esculenta* outside the area of clone formation: the view from protein electrophoresis. *J. Zool. Syst. Evol. Res.* **46**: 56–62.

Jakob, C., Arioli, M. & Reyer, H.-U. 2010. Ploidy composition in all-hybrid frog populations in relation to ecological conditions. *Evol. Ecol. Res.* **12**: 633–652.

Janko, K., Kotusz, J., De Gelas, K., Slechtova, V., Opoldusova, Z., Drozd, P. *et al.* 2012. Dynamic formation of asexual diploid and polyploid lineages: multilocus analysis of Cobitis reveals the mechanisms maintaining the diversity of clones. *PLoS One* **7**: e45384.

Kearney, M., Fujita, M.K. & Ridenour, J. 2009. Lost sex in the reptiles: constraints and correlations. In: *Lost Sex. The Evolutionary Biology of Parthenogenesis* (I. Schön, K. Martens & van Dijk P., eds), pp. 447–474. Springer, Heidelberg.

Lamatsch, D.K. & Stöck, M. 2009. Sperm-dependent parthenogenesis and hybridogenesis in teleost fishes. In: *Lost Sex. The Evolutionary Biology of Parthenogenesis* (I. Schön, K. Martens & van Dijk P., eds), pp. 399–432. Springer, Heidelberg.

Maynard-Smith, J. 1992. Age and the unisexual lineage. *Nature* **356**: 661–662.

Mikulíček, P. & Kotlík, P. 2001. Two water frog populations from western Slovakia consisting of diploid females and diploid and triploid males of the hybridogenetic *Rana esculenta* (Anura, Ranidae). *Mitt. Zool. Mus. Berlin* **77**: 59–64.

Mikulíček, P., Kautman, M., Kautman, J. & Pruvost, N.B.M. 2014b. Mode of hybridogenesis and habitat preferences influence population composition of water frogs (*Pelophylax esculentus* complex, Anura: Ranidae) in a region of sympatric occurrence (western Slovakia). *J. Zool. Syst. Evol. Res.* doi: 10.1111/jzs.12083.

Mikulíček, P., Kautman, M., Demovič, B. & Janko, K. 2014a. When a clonal genome finds its way back to a sexual species: evidence from ongoing but rare introgression in the hybridogenetic water frog complex. *J. Evol. Biol.* **27**: 628–642.

Moritz, C., Brown, W.M., Densmore, L.D., Wright, J.W., Vyas, D., Donnellan, S. *et al.* 1989. Genetic diversity and the dynamics of hybrid parthenogenesis in *Cnemidophorus* (Teiidae) and *Heteronotia* (Gekkonidae). In: *Ecology and Evolution of Unisexual Vertebrates*, Vol. 466 (R.M. Dawley & J.P. Bogart, eds), pp. 87–112. New York State Museum, Albany, NY.

Neaves, W.B. & Baumann, P. 2011. Unisexual reproduction among vertebrates. *Trends Genet.* **27**: 81–88.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance for small number of individuals. *Genetics* **89**: 583–590.

Otto, S.P. & Lenormand, T. 2002. Resolving the paradox of sex and recombination. *Nat. Rev. Genet.* **3**: 252–261.

Parker, E.D. Jr & Niklasson, M. 1999. Genetic structure and evolution in parthenogenetic animals. In: *Evolutionary Genetics: from Molecules to Morphology* (R.S. Singh, C.B. Krimbas, eds), pp. 456–474. Cambridge University Press, Cambridge.

Peakall, R. & Smouse, P.E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6**: 288–295.

Plötner, J. 2005. *Die westpaläarktischen Wasserfrösche*. Laurenti-Verlag, Bielefeld.

Polls Pelaz, M. 1994. Modes of gametogenesis among kleptons of the hybridogenetic water frog complex: an evolutionary synthesis. *Zool. Pol.* **39**: 123–128.

Pritchard, J.K., Stephens, M. & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Pruvost, N.B.M., Hoffmann, A. & Reyer, H.-U. 2013a. Gamete production patterns, ploidy, and population genetics reveal evolutionary significant units in hybrid water frogs (*Pelophylax esculentus*). *Ecol. Evol.* **3**: 2933–2946.

Pruvost, N.B.M., Hollinger, D. & Reyer, H.-U. 2013b. Genotype-temperature interactions on larval performance shape population structure in hybridogenetic water frogs (*Pelophylax esculentus* complex). *Funct. Ecol.* **27**: 459–471.

Rybacki, M. & Berger, L. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. *Mitt. Mus. Nat. Berlin, Zool. Reihe* **77**: 51–77.

Schmeller, D.S., Seitz, A., Crivelli, A. & Veith, M. 2005. Crossing species' range borders: interspecies gene exchange mediated by hybridogenesis. *Proc. Biol. Sci.* **272**: 1625–1631.

Schmidt, D.J., Bond, N.R., Adams, M. & Hughes, J.M. 2011. Cytonuclear evidence for hybridogenetic reproduction in natural populations of the Australian carp gudgeon (*Hypseleotris*: Eleotridae). *Mol. Ecol.* **20**: 3367–3380.

Schön, I., Martens, K. & van Dijk, P., eds. 2009. *Lost Sex. The Evolutionary Biology of Parthenogenesis*. Springer, Heidelberg.

Sinclair, E.A., Pramuk, J.B., Bezy, R.L., Crandall, K.A. & Sites, J.W. Jr 2010. DNA evidence for nonhybrid origins of parthenogenesis in natural populations of vertebrates. *Evolution* **64**: 1346–1357.

Som, C. & Reyer, H.-U. 2006. Demography and evolution of pure hybridogenetic frog (*Rana esculenta*) populations. *Evol. Ecol. Res.* **8**: 1235–1248.

Stenberg, P. & Saura, A. 2009. Cytology of asexual animals. In: *Lost Sex. The Evolutionary Biology of Parthenogenesis* (I. Schön, K. Martens, P. van Dijk, eds), pp. 63–74. Springer, Heidelberg.

Stöck, M., Lampert, K.P., Moeller, D., Schlupp, I. & Schartl, M. 2010a. Monophyletic origin of multiple clonal lineages in an asexual fish (*Poecilia formosa*). *Mol. Ecol.* **19**: 5204–5215.

Stöck, M., Ustinova, J., Lamatsch, D.K., Schartl, M., Perrin, N. & Moritz, C. 2010b. A vertebrate reproductive system involving three ploidy levels: hybrid origin of triploids in a contact zone of diploid and tetraploid Palearctic green toads (*Bufo viridis* subgroup). *Evolution* **64**: 944–959.

Stöck, M., Ustinova, J., Betto-Colliard, C., Schartl, M., Moritz, C. & Perrin, N. 2012. Simultaneous Mendelian and clonal genome transmission in a sexually reproducing, all-triploid vertebrate. *Proc. Biol. Sci.* **279**: 1293–1299.

Suomalainen, E., Saura, A. & Lokki, J. 1987. *Cytology and Evolution in Parthenogenesis*. CRC Press, Boca Raton, FL.

Tunner, H.G. 1974. Die klonale Struktur einer Wasserfroschpopulation. *Z. Zool. Syst. Evol.* **12**: 309–314.

Tunner, H.G. 1980. Kreuzungsexperimente mit Wasserfröschen aus österreichischen und polnischen Mischpopulationen (*Rana lessonae* + *Rana esculenta*). *Z. Zool. Syst. Evol.* **18**: 257–297.

Tunner, H.G. 2000. Evidence for genomic imprinting in unisexual triploid hybrid frogs. *Amphib.-Reptil* **21**: 135–141.

Tunner, H.G. & Dobrowsky, M.T. 1976. Morphological, serological and enzymological differentiation of *Rana lessonae* and hybridogenetic *Rana esculenta* from Seewinkel and Neusiedlersee (Austria, Burgenland). *Zool. Anz.* **197**: 6–22.

Tunner, H.G. & Heppich-Tunner, S. 1992. A new population system of water frogs discovered in Hungary. In: *Proc. 6th Ord. Gen. Meet. Soc. Europ. Her.* pp. 453–460. 19–23 August 1991. Budapest, Hungary.

Uzzell, T. & Berger, L. 1975. Electrophoretic phenotypes of *Rana ridibunda*, *Rana lessonae*, and their hybridogenetic associate, *Rana esculenta*. *Proc. Acad. Nat. Sci. Philadelphia* **127**: 13–24.

Uzzell, T., Berger, L. & Günther, R. 1975. Diploid and triploid progeny from a diploid female of *Rana esculenta* (Amphibia Salientia). *Proc. Acad. Nat. Sci. Philadelphia* **127**: 81–91.

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. 2004. Microchecker: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**: 535–538.

Vinogradov, A.E., Borkin, L.J., Günther, R. & Rosanov, J.M. 1990. Genome elimination in diploid and triploid *Rana esculenta* males – Cytological evidence from DNA flow cytometry. *Genome* **33**: 619–627.

Vorburger, C. 2001a. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. *Evolution* **55**: 2319–2332.

Vorburger, C. 2001b. Non-hybrid offspring from matings between hemiclonal hybrid water frogs suggest occasional recombination between clonal genomes. *Ecol. Lett.* **4**: 628–636.

Vrijenhoek, R. 1979. Factors affecting clonal diversity and coexistence. *Am. Zool.* **19**: 787–797.

Vrijenhoek, R.C. 1989. Genetic and ecological constraints on the origins and establishment of unisexual vertebrates. In: *Evolution and Ecology of Unisexual Vertebrates*, Vol. 466 (R.M. Dawley & J.P. Bogart, eds), pp. 24–31. New York State University Museum Bulletin, Albany, NY.

Vrijenhoek, R.C. 1999. Parthenogenesis and natural clones. In: *Encyclopedia of Reproduction*, Vol. 3 (E. Knobil & J.D. Neill, eds), pp. 695–702. Academic Press, San Diego, CA.

Vrijenhoek, R.C., Dawley, R.M., Cole, C.J. & Bogart, J.P. 1989. A list of the known unisexual vertebrates. In: *Evolution and Ecology of Unisexual Vertebrates*, Vol. 466 (R.M. Dawley & J.P. Bogart, eds), pp. 19–23. New York State University Museum Bulletin, Albany, NY.

Waits, L.P., Luikart, G. & Taberlet, P. 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Mol. Ecol.* **10**: 249–256.

Weir, B.S. & Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Zeisset, I., Rowe, G. & Beebee, T.J.C. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. *Mol. Ecol.* **9**: 1173–1174.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 DNA microsatelitte data file for the 16 loci used in the study (RlCA5 and Res16 were not used for any of the analyses).

Data S2 Genome sizes measured by flow cytometry based on blood (b) and sperm (s) samples.

Data S3 Gene diversity (H_e) corrected for sample size (Nei, 1978) for *P. lessonae* and *P. ridibundus* genomes in sexual and hybrid genotypes sampled in populations.

Data S4 Hemiclonal and single multilocus genotypes (MLGs) in four genotypes (LL, LR, LLR and RR) from nine study populations (Pop.).

Data S5 Three not mutually exclusive hypotheses to explain how diploid LR hybrids are maintained in populations types PT3 (LR females and LLR males) and PT4 (LR males and females, LLR males and RR males and females).

Data deposited at Dryad: doi:10.5061/dryad.hn4tn

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